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Research article

Abscisic acid mediated differential growth responses of root and shoot of *Vigna radiata* (L.) Wilczek seedlings under water stress

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ABSTRACT

Water stress is a common problem in Indian agriculture and recent global climate change has aggravated this problem further. Plants have an adaptive response to water scarcity as reflected in differential root and shoot growth. In case of *Vigna radiata* seedlings we have observed earlier promotion of root growth while inhibition of shoot (hypocotyl) growth under mild water stress, which is probably mediated by ABA. However, the exact mechanism of ABA action is far from clear. The present study attempts to elucidate the action of ABA through apoplastic reactive oxygen species (ROS) and its impact upon antioxidant defence system during the growth of root and shoot under water stress. Thus promotion of root growth by application of exogenous ABA (10 μM) and mild water stress (ψ −0.5 MPa) may be correlated with enhanced apoplastic ROS production possibly by activating plasma membrane located NADPH oxidase (NOX) enzyme. On the contrary, in hypocotyls where growth was rather inhibited by application of water stress or ABA, neither NOX activity nor ROS accumulation was significantly detected upon these treatments. Overall activity of antioxidant enzymes [superoxide dismutase (SOD, EC 1.15.1.1), peroxidase (POX, EC 1.11.1.7), ascorbate peroxidase (APX, EC 1.11.1.1) and catalase (CAT, EC 1.11.1.6)] was high and somewhat promoted by ABA and water stress treatment in roots compared to hypocotyls. A possible ROS-mediated role of ABA in promoting growth and antioxidant activity in roots under water stress has been proposed.

1. Introduction

Plant growth is a vulnerable process being affected by several factors under natural conditions, among which water deficit is one of the major factors. The productivity of crop plants reduces up to 80% of total production on average when plants are exposed to drought conditions in the field (Shao et al., 2009). Recent global climate changes aggravated this situation further leading to a greater loss of crop production. The adaptation to water deficit may arise either due to the ability to tolerate water deficit or from mechanisms that allow avoidance of the water deficit. Plants often can maintain water status by closing stomata as a rapid response to water deficit, while another option is elongation of root system as slower developmental response. Under laboratory condition water stress simulated by polyethylene glycol (PEG 6000) solution generally inhibits both root and shoot growth of plants, root growth being less inhibited compared to shoot growth (Spollen et al., 1993). However, low level of water stress (mild stress) has been found to promote root growth even over control (Das and Kar, 2013). Mild water stress induced root growth promotion and shoot growth inhibition is being considered as one of the adaptive mechanisms of plants to survive under water limited condition. The exact mechanisms of differential growth responses of root and shoot under water stress are not clearly known.

Abscisic acid (ABA) has been demonstrated to be associated with water stress being accumulated at high concentration and proposed to be involved in the regulation of water stress induced adaptive responses including growth (Wani and Kumar, 2015). The exact mechanism of ABA during mild water stress induced differential growth responses of plant is, however, far from clear till date, although some other factors may also be responsible for differential growth responses of plant under water stress (Ober and Sharp, 2007).

During last two decades the role of reactive oxygen species (ROS)

Abbreviations: Flu, fluridone; PEG, polyethylene glycol 6000; ROS, reactive oxygen species; XTT, 2,3-Bis(2-methoxy-4-nitro-5-sulfophenyl)-2*H*-tetrazolium-5-carboxanilide inner salt; NOX, NADPH oxidase; SOD, superoxide dismutase; POX, peroxidase; APX, ascorbate peroxidase; CAT, catalase; $O_2^{\cdot-}$, superoxide; H_2O_2, hydrogen peroxide; $OH^{\cdot}$, hydroxyl radical; PAGE, polyacrylamide gel electrophoresis; NBT, nitroblue tetrazolium chloride; TEMED, tetramethylethylenediamine; NaN_3, sodium azide; DTT, 1,4-dithiothreitol; EDTA, ethylenediaminetetraacetic acid; PVP, polyvinyl pyrrolidone; DAB, diaminobenzidine; $K_3Fe(CN)_6$, potassium ferricyanide; $FeCl_3$, ferric chloride; DPI, diphenylene iodonium chloride; TMB, 3,3′,5,5′-tetramethyl benzidine dihydrochloride hydrate

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has been extensively studied on plant growth and development. ROS are as such highly reactive and their overproduction causes cellular damage or even cellular death (Apel and Hirt, 2004). Under normal condition ROS, being produced in several biochemical reactions, are scavenged by plants own antioxidant system, but only becomes deleterious when production exceeds scavenging capacity. However, in recent past ROS are being implicated for having a positive role in plant growth and development as well as plant adaptation to biotic and abiotic stress (Singh et al., 2014). In such cases, initially superoxide ($O_2^{\cdot-}$) formation occurs in cell wall due to activity of plasma membrane located NADPH oxidase (NOX). Subsequently $O_2^{\cdot-}$ is converted to less reactive H_2O_2 spontaneously or via the action of superoxide dismutases (SOD) (Kar, 2011). H_2O_2 may further be converted to hydroxyl radical ($OH^{\cdot}$), which is proposed to be involved in the scission of cell wall polysaccharides effecting wall loosening and extension growth (Singh et al., 2015). Thus it appears that generation of a minimal level of ROS is also indispensible for growth and development of plants under water stress condition (Gill and Tuteja, 2010; Das and Kar, 2016).

On the other hand, plants have evolved a number of antioxidant enzymes and antioxidant molecules that are being used to ameliorate oxidative stress by scavenging toxic oxygen species (Liang et al., 2003). Such antioxidant system comprises low-molecular mass antioxidants (glutathione, ascorbate, carotenoids) and ROS-scavenging enzymes [superoxide dismutase (SOD, EC 1.15.1.1), peroxidase (POX, EC 1.11.1.7), catalase (CAT, EC 1.11.1.6), ascorbate peroxidase (APX, EC 1.11.1.1)] (Apel and Hirt, 2004). Eventually, an increased level of antioxidative capacity under water stress can be considered as an important measure for drought tolerance (Dawood et al., 2014). Therefore, it is expected that apoplastic ROS that initiates extension growth through cell wall loosening, may also be effective somehow in inducing antioxidative protection against ROS-induced damages during root elongation under mild water stress.

Present study aimed to get an insight of how ABA mediates differential growth responses in root and shoot by affecting apoplastic ROS and antioxidant defence system under water stress.

2. Material and methods

2.1. Plant material, incubation and growth measurement

Early stage grown mung bean [*Vigna radiata* (L.) Wilczek var. B1] seedlings were used as the experimental material. Seeds were first surface sterilized by 1% sodium hypochlorite solution followed by the twice rinsing in distilled water and finally incubated on the moistened Whatman No. 1 filter paper placed in a 9 cm diameter Petri dish and kept in a Seed Germinator at 30 °C (± 2 °C) for 20 h. After 20 h, the germinated seeds were transferred into transparent (for light treatment) and black coated (for dark treatment) plastic boxes containing Whatman No. 1 filter paper which was soaked with either distilled water (control) or test solutions under stress (mild water stress, $\psi = -0.5$ MPa) or non stress condition (dH_2O). Mild water stress was created by using particular concentration of polyethylene glycol (19.6% PEG 6000) solution (Michel and Kaufmann, 1973). The test solutions were abscisic acid (ABA 10 μM) and its biosynthetic inhibitor fluridone (10 μM). Optimum concentrations for the test solutions were chosen according to the growth response of seedlings in a range of different concentrations tested under non stress condition. Water potential of $\psi = -0.5$ MPa was considered as mild water stress, since effect of this level of stress on seedlings was not severe. In all the treatments, growth measurement of seedlings was done by measuring root (radicle) and shoot (hypocotyl) length at one day intervals up to 3 days. Plastic boxes (both transparent as well as black coated) were kept in a Seed Germinator. Incubation for root growth (length in mm) was done always under continuous dark condition (black coated plastic box) while that for shoot growth (length in mm) was under continuous light (transparent plastic box) condition (150 μmol m^{-2} s^{-1}), since under natural conditions root system remains buried in the soil (equivalent to darkness) and shoots are exposed to air (light). Such differential sampling i.e. dark incubation for root studies and light incubation for shoot studies have also been followed in case of other respective analyses.

2.2. ABA and PEG induced extracellular ROS production

2.2.1. Superoxide ($O_2^{\cdot-}$) production

Extracellular $O_2^{\cdot-}$ production in root and hypocotyl tissues of seedlings, grown under treatment with ABA (10 μM), PEG ($\psi = -0.5$ MPa) and control condition, was measured following the method of Das and Kar (2016). After excision of three day old seedlings, roots and hypocotyls were kept separately in distilled water for 10 min for release of wound induced $O_2^{\cdot-}$. After that root and hypocotyl tissues (300 mg each) were immersed in a medium comprising 500 μl of 0.05M sodium phosphate buffer (pH 6.8), 250 μl of distilled water and 250 μl of XTT (2,3-Bis(2-methoxy-4-nitro-5-sulfophenyl)-2*H*-tetrazolium-5-carboxanilide inner salt) solution (final concentration 0.5 mM) in 1.5 ml eppendorf tubes and kept on a shaker for 45 min at 35–37 °C in an incubator. Then absorbance of the bathing medium was recorded at 470 nm in UV–visible spectrophotometer (Systronics, India).

2.2.2. $O_2^{\cdot-}$ staining in roots and hypocotyls

Superoxide ($O_2^{\cdot-}$) accumulation in roots and hypocotyls of normal, ABA (10 μM) and mild stress grown seedlings was determined by staining with NBT (0.5 mM) in sodium phosphate buffer (50 mM, pH 6.8) (Das and Kar, 2016). Root and hypocotyl tissues, after excision from intact seedlings, were first kept in distilled water for 10 min to release extra $O_2^{\cdot-}$, which might have been produced due to injury. Then these tissues were blotted to soak surface water and dipped in NBT (0.5 mM) solution at 30 °C. After 30 min stained roots and hypocotyls were washed with distilled water and photographed using CANON (Power Shot A640) camera.

2.2.3. Hydrogen peroxide (H_2O_2) production

Extracellular H_2O_2 production in root and shoot (hypocotyl) tissues was determined spectrophotometrically using xylenol orange (Das and Kar, 2016). Bathing medium used was a mixture of 1 part of Reagent A containing 25 mM $FeSO_4$, 25 mM $(NH_4)_2SO_4$ and 2.5 M H_2SO_4 and 100 parts of Reagent B containing 125 μM xylenol orange and 100 mM sorbitol. Three replicates of equal amount (300 mg) of root and shoot (hypocotyl) tissues of control, mild water stress and ABA (10 μM) grown seedlings of *V. radiata* were immersed in the above 1.5 mL of bathing medium and gently shaken at 60 rpm for 30 min in the dark at 25 °C followed by absorbance measurement of the assay mixture at 560 nm in UV–visible spectrophotometer (Systronics, India). H_2O_2 production was calculated using a standard curve with known concentrations of H_2O_2.

2.2.4. H_2O_2 staining in roots and hypocotyls

Hydrogen peroxide (H_2O_2) accumulation in roots and hypocotyls of normal, ABA (10 μM) and mild water stress grown seedlings was localized by H_2O_2 specific stain TMB (3,3′,5,5′-Tetramethyl benzidine dihydrochloride hydrate). Seedlings of control, ABA and mild water stress grown sets were excised into roots and hypocotyls, which were then kept in distilled water for 10 min to release excess ROS, as mentioned earlier. After that these tissues were blotted to soak surface water and dipped in TMB solution (1 mM) at 30 °C for 30 min and then washed with distilled water and photographed using CANON (Power Shot A640) camera.

2.3. Analysis of membrane bound NADPH oxidase (NOX) enzyme activity of control, ABA and PEG-treated seedlings

2.3.1. Isolation of membrane fraction

Membrane fraction was isolated following the method of Hejl and

Koster (2004) after modification by Das and Kar (2016). Root or hypocotyl tissues were homogenized in a medium containing 50 mM sodium phosphate buffer (pH 6.8), sucrose (250 mM), EDTA (Ethylene diamine tetraacetic Acid) (3 mM), DTT (Dithiothreitol) (1.25 mM) and PMSF (Phenylmethane sulphonyl flouride) (1 mM) and centrifuged at 10,000g for 10 min followed by centrifugation of the supernatant at 25,000g for 45 min to obtain membrane fraction. Membrane fraction was then resuspended in 50 µl resuspention medium containing 50 mM sodium phosphate buffer (pH 6.8), sucrose (250 mM), Triton X-100 (0.5%), DTT (1 mM), PMSF (1 mM), kept in ice for 1 h and centrifuged again at 25,000g for 45 min. Supernatant was collected and used as enzyme source for NOX assay.

2.3.2. In-gel assay of NOX

NOX activity was determined in 7.5% (1 mm) vertical native PAGE at 4 °C. After quantifying the protein contents using Bradford reagent (Bradford, 1976), protein samples (15 µg) from each treatment were mixed with loading buffer and run on a vertical native PAGE at 4 °C in a Mini Cold Lab (LKB, Bromma, Sweden). The activity of NOX was detected in the gel strips by incubating in reaction medium containing Tris-HCl buffer (10 mM, pH 7.4), NADPH (0.2 mM), $MgCl_2$ (0.1 mM), $CaCl_2$ (1 mM) and 1 mM NBT (Carter et al., 2007). Blue-violet coloured bands appeared due to formation of insoluble formazan as a result of the reduction of NBT by $O_2^{\cdot -}$ generated from NOX activity. For confirmation diphenylene iodonium chloride (DPI 0.2 mM) was used in the incubation medium, as DPI is a potent inhibitor of NOX. Gel photographs were taken using a CANON (Power Shot A640) camera.

2.4. Analysis of superoxide dismutase (SOD) activity of control, ABA and PEG-treated seedlings

SOD activity was studied by in gel assay carried out with 10% resolving gel and 5% stacking gel. Root and hypocotyl tissues were homogenized in 0.15 M Tris buffer (pH 7.5) under chilling conditions (Chen and Pan, 1996) and centrifuged at 10,000 g at 4 °C. Supernatant, used as protein sample (40 µg), from each treatment was mixed with loading buffer and run on a vertical native PAGE at 4 °C in standard Tris-Glycine tank buffer (pH 8.3). Activity of SOD was determined by modified photochemical method of Beauchamp and Fridovich (1971). The gel was first soaked in 25 ml of 1.23 mM NBT for 15 min, briefly washed, then soaked in 30 ml of 100 mM potassium phosphate buffer (pH 7.0) containing 28 mM TEMED and 2.8×10^{-2} mM riboflavin for another 15 min in the dark. The gel was briefly washed again and then illuminated in a light box (200 µmol m^{-2} s^{-1}) for 15 min to initiate the photochemical reaction. SOD activity was detected as achromatic bands upon a background of blue-violet colour. Different isoforms of SOD were identified by inhibition test using H_2O_2 (10 mM) and sodium azide (NaN_3, 5 mM) (data not shown).

2.5. Analysis of peroxidase (POX) activity of control, ABA and PEG-treated seedlings

POX activity was determined in 7.5% non-denaturing gel in standard Tris-Glycine tank buffer (pH 8.4). Root and hypocotyl tissues were homogenized in 100 mM Tris-HCl buffer (pH 7.5) containing 1.25 mM DTT, 3 mM EDTA and 2% polyvinyl pyrolidone (PVP). Homogenates were centrifuged at 10,000 g at 4 °C for 10 min and supernatants were used for in gel assay of peroxidase activity (Prodanovic et al., 2007). The protein samples (15 µg) from each treatment were mixed with loading buffer and run on a vertical native PAGE at 4 °C. The gel was stained following the method of Sabar et al. (2005) with few modifications. The gel slab was immersed in 0.05M sodium acetate buffer (pH 5.5) containing 0.1% DAB (diaminobenzidine) and 5 mM H_2O_2. Brown colour bands appeared in the gel corresponding to peroxidase isozymes. To confirm peroxidase activity the gel was also stained in 0.1% DAB in presence of 5 mM SHAM (salicylhydroxamic acid), a POX inhibitor (data not shown).

2.6. Analysis of ascorbate peroxidase (APX) activity of control, ABA and PEG-treated seedlings

APX activity was determined in 7.5% (1 mm) vertical native PAGE at 4 °C. Root and hypocotyl tissues were homogenized in extraction buffer containing 100 mM Tris (pH 7.5), 1 mM EDTA, 0.5% Triton X 100, 1 mM DTT and 2% PVP and centrifuged at 10,000g for 10 min (Prodanovic et al., 2007). The supernatant was used as enzyme source. The samples were mixed with 5X gel loading buffer and loaded in respective wells (40 µg protein) and the gel was run at a constant voltage of 90 V for 4 h at 4 °C. After run the gel slab was incubated for 15 min at room temperature under agitation in 0.1M Na-phosphate buffer (pH 6.2) containing 4 mM ascorbic acid and 4 mM H_2O_2. The gel slab was then stained with a solution of 0.125M HCl containing 0.1% (final concentration) potassium ferricyanide [$K_3Fe(CN)_6$] and 0.1% (final concentration) ferric chloride ($FeCl_3$). APX was localized as an achromatic band on a Prussian blue background.

2.7. Analysis of catalase (CAT) activity of control, ABA and PEG treated seedlings

CAT activity was determined in 7.5% non-denaturing gel in standard Tris-Glycine tank buffer (pH 8.4) at 4 °C. Tissues were homogenized in 50 mM phosphate buffer (pH 7.5) containing 1 mM EDTA, 1 mM DTT and 4% PVP (Garnczarska and Wojtyla, 2008). Homogenates were centrifuged at 10,000g for 15 min and supernatants were used as enzyme source. The samples were mixed with 5X gel loading buffer and loaded in respective wells (50 µg protein). Staining procedure was adopted from Prodanovic et al. (2007) with few modifications. The gel slab was incubated in 10 mM H_2O_2 dissolved in 50 mM sodium acetate buffer (pH 5.5) for 20 min followed by incubation in a mixture of 1% (final) $K_3Fe(CN)_6$ and 1% (final) $FeCl_3$ for 15 min. Achromatic bands corresponding to CAT enzyme appeared in dark green coloured gel.

2.8. Statistical analysis

In case of growth data final results were presented as average length of root and hypocotyl from 10 seedlings. Data were statistically analysed by subjecting to ANOVA and probability values were determined for significance. Standard errors (SE) around the mean value were shown in the figures as vertical error bars (Clarke, 1969). In case of in gel assay each assay was repeated thrice.

3. Result

3.1. Role of ABA on root (radicle) and shoot (hypocotyl) growth of non-stress and stress grown seedlings

Germinated seeds of *Vigna radiata* were treated with ABA (10 µM) and Fluridone (10 µM), an ABA biosynthesis inhibitor, results of which were depicted in Fig. 1. There was a promotion of root growth in ABA treated seedling under non-stress condition, while exogenous application of ABA somewhat inhibited root growth under mild water stress condition (Fig. 1a). In case of shoot (hypocotyl) growth, exogenous ABA application marginally inhibited normal shoot growth compared to PEG-induced severe growth inhibition, whereas exogenous ABA has no effect on water stress induced severe shoot growth inhibition (Fig. 1b). On the other hand, fluridone severely inhibited normal as well as water stress induced promotion of root growth (Fig. 1c). In contrast, fluridone significantly promoted normal shoot growth and significantly recovered stress induced severe shoot growth inhibition (Fig. 1d).

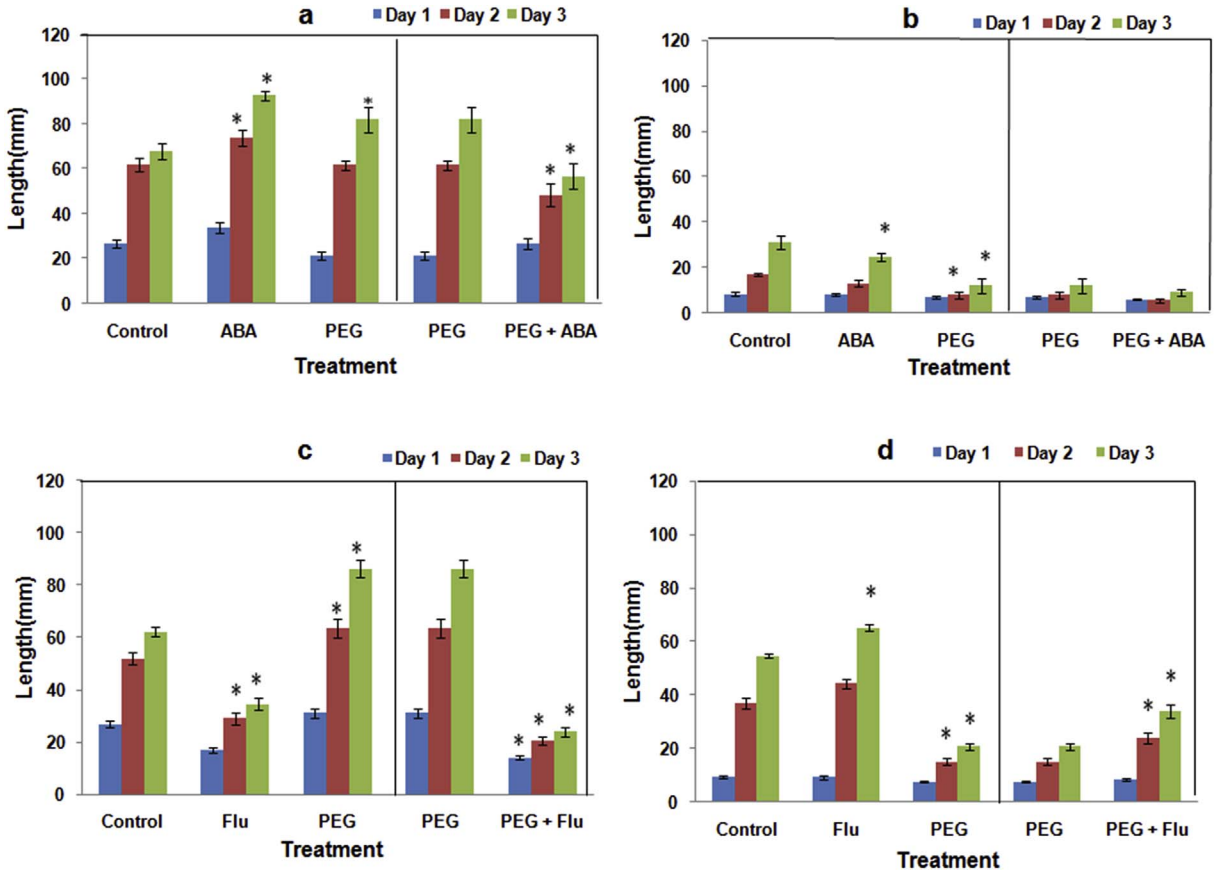

Fig. 1. Role of ABA in seedling growth of *Vigna radiata* under water stress and non-stress condition. Germinated seeds were incubated in presence of ABA (10 μM) under non-stress as well as PEG 6000 induced mild water stress (ψ = −0.5 MPa) and length of root (a) and hypocotyl (b) was measured at 1 day intervals up to 3 days. Root (c) and hypocotyl (d) length was also measured in fluridone (Flu 10 μM) treated seedlings under normal as well as PEG-induced stress (- 0.5 MPa) condition. Data were subjected to one way ANOVA. Asterisk (*) indicates a significant difference among means at $P < 0.05$ level between control and test set. Significance was determined for the left group of data (left box) with respect to non-stress control and for right group of data (right box) with respect to PEG-treated control. SE shown as vertical bars.

3.2. ABA and water stress-induced extracellular ROS production and activation of NADPH oxidase (NOX) enzyme

3.2.1. Extracelluar $O_2^{\bullet -}$ production

Production of extracellular $O_2^{\bullet -}$ during seedling growth under normal, water stress and ABA treatment (10 μM) was monitored by analyzing $O_2^{\bullet -}$ level in the bathing medium of roots and hypocotyls using XTT. It was observed that extracellular production of $O_2^{\bullet -}$ was quite high in roots under water stress and ABA treatment compared to normal grown roots (Fig. 2a). In case of hypocotyls, extracellular $O_2^{\bullet -}$ production was very low compared to roots. Moreover, although marginal increase of $O_2^{\bullet -}$ production was observed under water stress, the level of $O_2^{\bullet -}$ declined to minimum under ABA treatment (Fig. 2a).

3.2.2. Extracellular H_2O_2 production

Extracellular H_2O_2 production under water stress, ABA and control grown roots and hypocotyls was monitored by analyzing H_2O_2 levels in the bathing medium using xylenol orange assay. Extracellular production of H_2O_2 was also high in roots treated with ABA and water stress as compared to control (Fig. 2b). In case of hypocotyls, extracellular H_2O_2 production was low in comparison with roots. However, H_2O_2 production was higher in hypocotyls treated with PEG and ABA than that in control (Fig. 2b).

3.2.3. Activity of NOX in roots and hypocotyls

In-gel assay (native PAGE) for NOX activity in roots and hypocotyls under control, water stress and ABA treated seedlings (Fig. 2c A & B) reveals that in roots NBT sensitive bands corresponding to NOX enzyme were prominent while in hypocotyls very faint bands appeared. Bands were unaffected by NaN_3 (an inhibitor of peroxidase) which was present in the incubation media. However, bands were sensitive to DPI, as revealed by disappearance of the bands in presence of DPI (Fig. 2c C & D). These bands were more intensified in case of roots under water stress as well as ABA compared to control (Fig. 2c A). On the other hand, in hypocotyls neither the bands were prominent nor these were intensified under ABA and stress grown seedlings (Fig. 2c B).

3.2.4. Staining of seedlings for $O_2^{\bullet -}$

Staining of roots and hypocotyls for $O_2^{\bullet -}$ production using NBT stain showed that roots were taking violet colour (due to reduction of tetrazolium salt by $O_2^{\bullet -}$) and such staining was more intense under ABA and water stress condition compared to control (Fig. 3a). On the other hand, hypocotyls did not take any colour under ABA, water stress as well as control condition (Fig. 3b).

3.2.5. H_2O_2 staining of seedlings

Hydrogen peroxide (H_2O_2) production in roots and hypocotyls of control, ABA and PEG treated seedling was observed using TMB staining. Results showed that roots of ABA and PEG treated seedlings took more intense blue colour compared to normal grown seedlings. However, hypocotyls of all the treatments did not take colour under both the conditions (Fig. 3c and d).

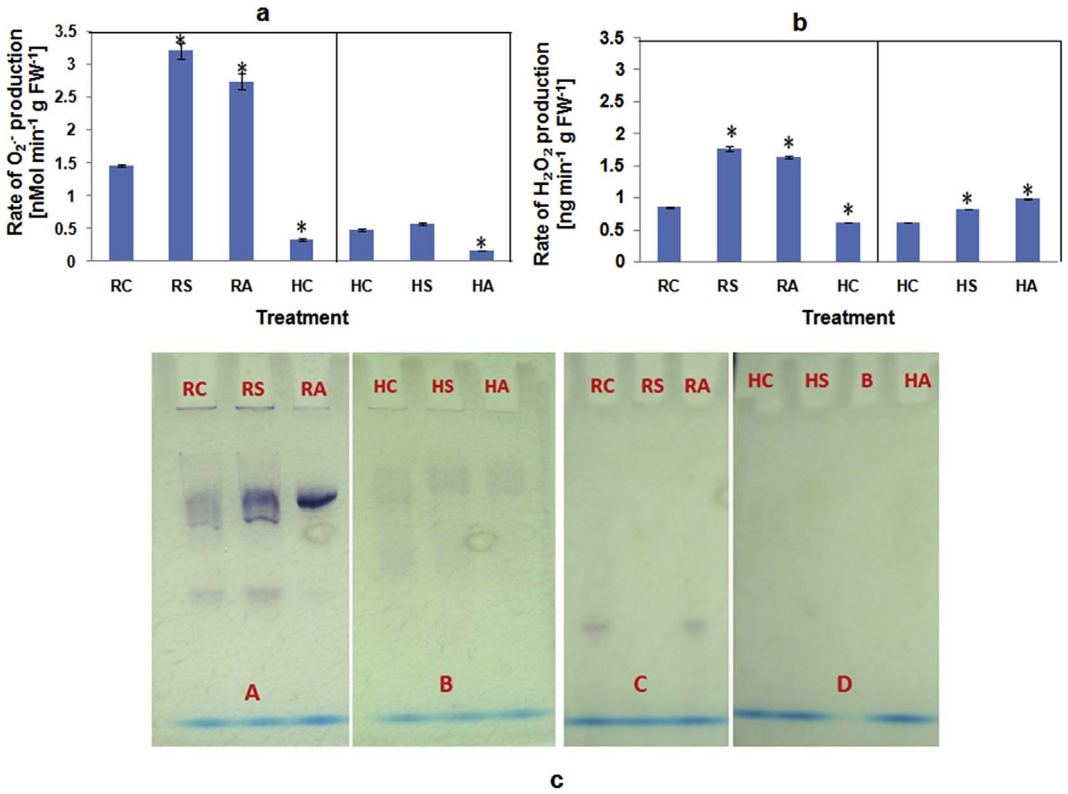

Fig. 2. (a & b) Extracellular superoxide ($O_2^{\cdot-}$) and hydrogen peroxide (H_2O_2) production in root (RC, RS, RA) and hypocotyl (HC, HS, HA) tissues from normal (RC, HC), water stress grown (RS, HS) and ABA (10 μM) treated (RA, HA) 3 days old seedlings. Superoxide ($O_2^{\cdot-}$) and hydrogen peroxide (H_2O_2) level was determined in the bathing medium of roots and hypocotyls by incubating with XTT (30 min) and xylenol orange (30 min), respectively. Data were subjected to one way ANOVA. Asterisk (*) indicates a significant difference among means at $P < 0.05$ level between control and test set. For determination of significance of difference, root control was considered in the left box for comparison with treated (both PEG and ABA) roots as well as unstressed hypocotyls, whereas in the right box hypocotyl control was considered for comparison with treated hypocotyls only. SE shown as vertical bars. (c) In-gel assay of membrane bound NADPH oxidase in root (A & C) and hypocotyls (B & D) of 3 day old seedlings. First lane (from left) of all the gels corresponds to control (RC, HC), second lane from left corresponds to stress (RS, HS) and third lane from the left corresponds to ABA (10 μM) (RA, HA) treatments. The gel was stained in presence of sodium azide (NaN_3, 1 mM). The gel was also incubated in presence of 0.2 mM DPI (a potent NOX inhibitor) for root (C) and shoot (D) tissues. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

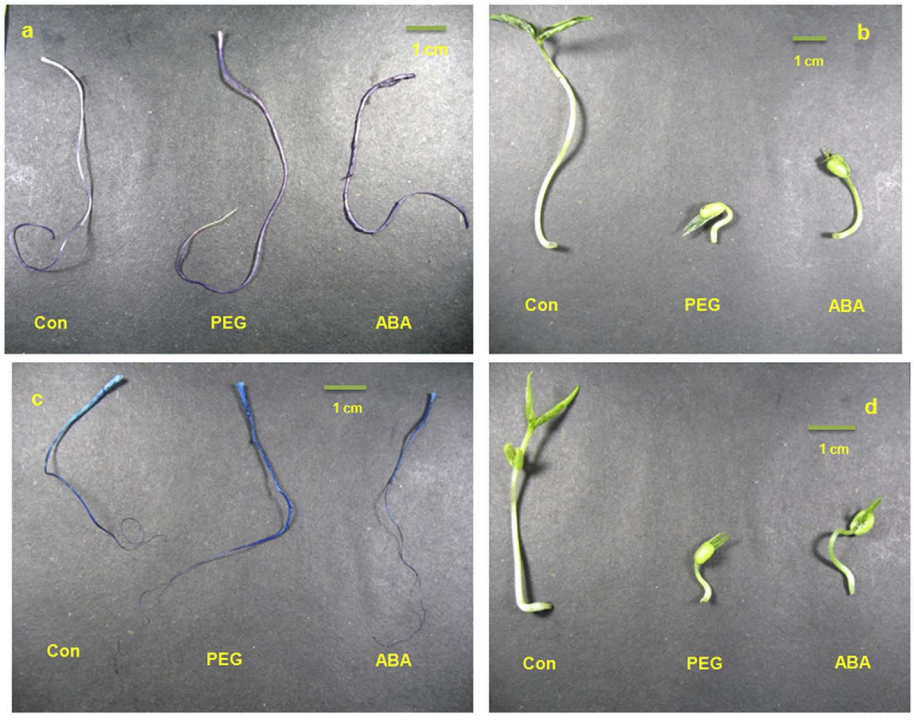

Fig. 3. Staining for superoxide ($O_2^{\cdot-}$) (a & b) and hydrogen peroxide (H_2O_2) (c & d) in roots (a & c) and hypocotyls (b & d) of *Vigna radiata* seedlings incubated under control, ABA (10 μM) and water stress condition using NBT and TMB, respectively. In all cases photographs are representatives of 15 replicates.

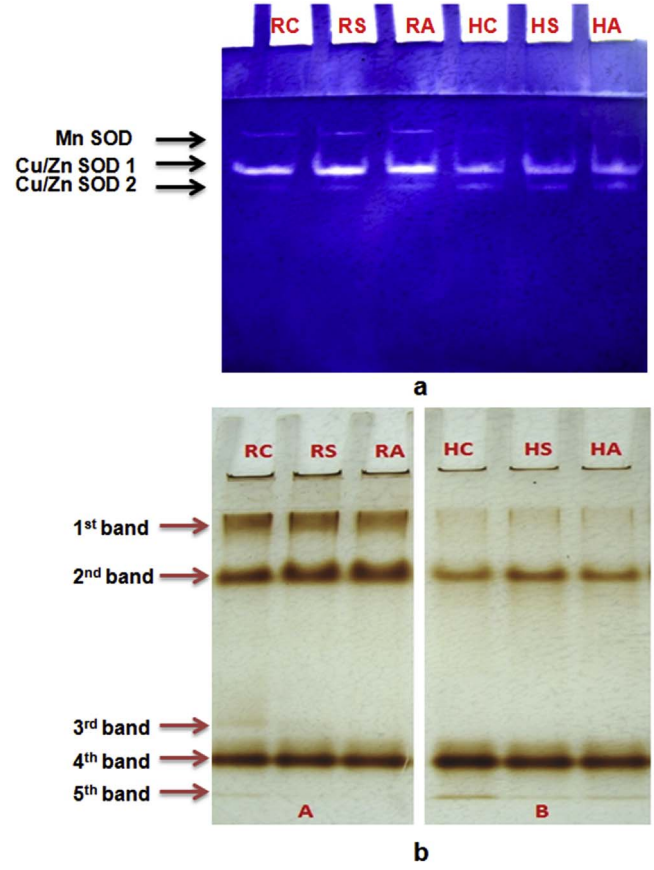

Fig. 4. In-gel assay of different superoxide dismutase (SOD) isoforms (a) and peroxidase (POX) isoforms (b) in root and hypocotyl tissues of 3 day old seedlings. (a) First and fourth lane (from left) corresponds to control of roots and hypocotyls (RC, HC), second and fifth lane corresponds to stress (RS, HS) and third and sixth lane corresponds to ABA (10 μM) (RA & HA) treatment. The first achromatic band (top of the gel) represents Mn SOD (the band was resistant to NaN_3 and H_2O_2), the second and third achromatic band represent Cu/Zn SOD 1 and Cu/Zn SOD 2, respectively (the bands were sensitive to NaN_3 and H_2O_2). (b) First lane (from left) of both the gels corresponds to control of roots and hypocotyls (RC, HC), second lane corresponds to stress (RS, HS) and third lane corresponds to ABA (10 μM) (RA & HA) treatment.

3.3. Effect of water stress and exogenous ABA on superoxide dismutase (SOD) and peroxidase (POX) activity

3.3.1. Activity and isoforms of SOD in roots and hypocotyls

In gel assay for SOD activity, done through inhibition of reduction of NBT, reveals three SOD isoforms detected in both root and hypocotyl tissues. Out of three isoforms, Cu/Zn SOD 1 and, to some extent, Mn SOD were prominent in root compared to hypocotyl tissues. On the other hand Cu/Zn SOD 2 was somewhat more prominent in hypocotyls (Fig. 4a). Moreover, in case of roots, an increase in the activity was found only for Cu/Zn SOD 1 under PEG and ABA treatments, whereas activity of Cu/Zn SOD 2 and Mn SOD changed a little. On the other hand, all these activities were almost unaltered under treatments in hypocotyl tissues.

3.3.2. Activity and isoforms of POX in roots and hypocotyls

POX activity was assessed through in gel assay by the reduction of DAB which was present in incubation medium of the gel. It was observed that five bands altogether became apparent in case of root tissues under control condition; out of these 1st, 2nd and 4th (from top) bands were intense while 3rd and 5th were very faint (Fig. 4b A). Upon exposure to stress and ABA treatment only 2nd band became more intense whereas 3rd and 5th band disappeared. On the other hand, hypocotyl tissues showed 1st and 2nd band, relatively fainter than those from roots, no 3rd band but a very dense 4th and a detectable 5th band (Fig. 4b B). After applying stress and exogenous ABA, 2nd band became thicker while 5th band became fainter having no change in the 1st and 4th band in case of hypocotyls.

3.4. Effect of water stress and exogenous ABA on ascorbate peroxidase (APX) and catalase (CAT) activity

3.4.1. Activity of APX in roots and hypocotyls

APX is one of the important antioxidant enzymes that breakdown H_2O_2 with the help of substrate ascorbate. APX activity was assayed in gel by observing the width of the achromatic band corresponding to ascorbate peroxidase enzyme. Results showed that under water stress APX activity increased in root tissues compared to control seedlings, while in ABA treated seedlings APX activity rather somewhat declined compared to control (Fig. 5a A). On the other hand, in case of hypocotyl tissues water stress treatment showed less APX activity compared to control and ABA-treated seedlings (Fig. 5a B).

3.4.2. Activity of CAT in roots and hypocotyls

Catalase activity was determined in gel (native PAGE assay) by observing the width of achromatic bands on dark green coloured gel. It was found that ABA and water stress treated seedlings showed increased catalase activity more prominently in roots but also in hypocotyl tissues (Fig. 5b A & B). However, in case of hypocotyls the bands appeared slightly at lower position compared to roots in the same gel slab. It may be due to different isoforms having different molecular weight of catalase enzymes in root and shoot tissues (Fig. 5b).

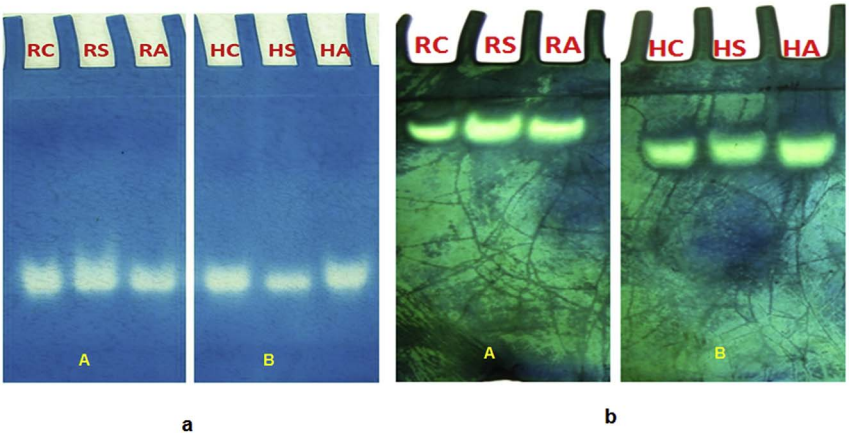

Fig. 5. In-gel assay of ascorbate peroxidase (APX) (a) and catalase (b) in root (A) and hypocotyl (B) tissues of 3 day old seedlings. First lane (from left) of the gels corresponds to control (RC, HC), second lane from left corresponds to stress (RS, HS) and third lane from the left corresponds to ABA (10 μM) (RA & HA) treatment.

4. Discussion

4.1. Role of abscisic acid (ABA) during root and shoot growth under stress

Abscisic acid (ABA), widely known as a stress hormone, generally accumulates in plant tissues at high concentration as a first hand response, when plants are exposed to environmental stress condition. In fact, stress tolerance is proposed to be associated with such accumulation of ABA that binds to its receptor to initiate signal transduction leading to up-regulation of stress response genes that help plants to cope with unsuitable environments (Ng et al., 2014). Practically, decreased root water potential in response to reduction in soil water potential causes ABA synthesis (Puértolas et al., 2013). Also, ABA has been reported to accumulate under water stress in different plant organs like leaves and vascular tissues, besides roots (Mittler and Blumwald, 2015). However, such accumulation in different organs may induce differential responses. As an adaptive response, water deficit often induces root growth while inhibiting shoot growth of plants, thus allowing for the exploration of surrounding soil environments for water but, at the same time, managing water loss from aerial parts under mild water stress. In the present study with *Vigna radiata* seedlings we have attempted to establish the role of ABA (possibly mediated via ROS) in differential growth responses of root and shoot under water stress, what we observed earlier (Das and Kar, 2016). Our observations that ABA mimics the effect of PEG-induced water stress by enhancing root growth but retarding hypocotyl growth (Fig. 1), which was reversed by blocking ABA biosynthesis (using fluridone), suggest the possible synthesis of ABA induced by water stress that might, in turn, promoted root growth at one hand, while inhibited shoot growth on the other. Similar observations have also been made earlier with maize seedlings, where increased endogenous ABA was implicated for shoot growth inhibition while continuing root growth under water stress (Saab et al., 1990; Sharp, 2002).

4.2. ABA and water stress induced extracellular ROS production

Most of the environmental stresses induce oxidative stress leading to ROS production and this mainly occurs due to increased photorespiration, oxidation of fatty acids and activity of the mitochondrial electron transport chain (mETC). Excess amount of ROS has potentially damaging effects on cellular components, but in very low amount ROS (may be in the extracellular space preferably) can also play vital roles in cellular signalling and in mediating plant responses to environmental stresses (Torres et al., 2005). Plant hormone, ABA can interact with both the production and signalling functions of ROS. ABA is a key inducer of H_2O_2 production as observed in the leaves of maize plants under water stress (Hu et al., 2006) and several studies have shown that ABA induced H_2O_2 production is involved in stomatal closure (Kwak et al., 2003; Desikan et al., 2004). It was also demonstrated that ABA signal transduction interacts with ROS metabolism both upstream and downstream of ROS production (Kwak et al., 2006). As was established earlier, apoplastic accumulation of $O_2^{\bullet-}$ and its metabolised product, H_2O_2 was associated with axis growth of germinating *V. radiata* seeds (Singh et al., 2014). A similar mechanism may be apprehended for the observed root growth of *V. radiata* seedlings under mild water stress that may be mediated through ABA. Evidently, water stress as well as ABA treatment induced higher rate of extracellular ROS accumulation (both $O_2^{\cdot-}$ and H_2O_2) as supported by spectrophotometric analysis (Fig. 2) and ROS-specific staining (Fig. 3).

However, in case of shoot (hypocotyls), where both water stress and ABA treatment inhibited growth, no accumulation of ROS (except a little accumulation of H_2O_2) was noticed. It appears that ABA has a different action in shoot system not mediated through ROS.

4.3. ABA and water stress induced NADPH oxidase (NOX) activity

NADPH oxidase (NOX) is a plasma membrane bound enzyme that catalyzes the production of superoxide ($O_2^{\cdot-}$) in apoplastic space by transferring electrons from NADPH to molecular oxygen. NADPH-oxidase generated ROS has been shown to play crucial roles in biotic interactions, abiotic stress and developmental processes including seed germination, seedling growth and root growth promotion under mild water stress in higher plants (Kwak et al., 2003; Carter et al., 2007; Singh et al., 2014; Das and Kar, 2016). It is also found that ABA promotes ROS production by plasma membrane-associated NADPH oxidases (Kwak et al., 2003).

Present study (in-gel assay for NOX activity) also indicates a strong correlation of NOX activity with water stress and ABA in case of roots only (Fig. 2c) suggesting a possibility of water stress-induced NOX activation mediated through ABA that, in turn, caused an accumulation of ROS in apoplast that resulted in cell wall relaxation required for growth. On the contrary, shoot growth inhibition could not be correlated with NOX-induced extracellular ROS production.

4.4. ABA and water stress induced antioxidant defence

As a consequence of water stress plant tissues often produce ROS through diverse cellular metabolism in the cell compartments, like chloroplasts, peroxisomes, mitochondria and cytosol (Cho et al., 2009). To overcome toxic effects of such ROS, plants have evolved antioxidant systems that ameliorate oxidative stress by scavenging toxic oxygen species (Liang et al., 2003). Thus, the degree of drought damage was apparently found to be negatively correlated with antioxidant enzymes like catalase (CAT), peroxidase (POX) and superoxide dismutase (SOD) activities, but corresponded positively with MDA (a lipid peroxidation product) accumulation (Liu et al., 2011). Present study revealed that exogenous ABA and water stress, though increased extracellular ROS production remarkably in roots (Figs. 2 and 3), significantly enhanced antioxidant defence potential at the same time by increasing the activity of different antioxidant enzymes. It may be apprehended that extracellular ROS initiated by NOX activity under water stress (mediated via ABA) in roots, in addition to inducing growth through enhanced cell wall relaxation (Singh et al., 2014), may be involved in signalling for activation of antioxidant enzyme network.

4.4.1. Superoxide dismutase (SOD) activity

Superoxide dismutase (SOD; EC 1.15.1.1), a metallo enzyme ubiquitous in all aerobic organisms, is the first line of defence against the toxic effects of high levels of ROS (Gill and Tuteja, 2010). Root tissues of mung bean seedlings showed an overall higher SOD activity (Fig. 4a). Among different isoforms of SOD, present in the seedlings, Mn SOD, prevalent in the mitochondrion and the peroxisome (Bresciani et al., 2014), showed higher activity in roots possibly indicating a better protection against ROS induced damages. However, enhanced activity of Cu/Zn SOD 1 activity, possibly located in the cell wall, under water stress and ABA treatment may be implicated for $OH^{\cdot}$-mediated cell wall relaxation required for water stress-induced elongation of root. Similar increment in Cu/Zn SOD 1 activity has already been demonstrated to be associated with radicle growth during seed germination of *Vigna radiata* (Singh et al., 2014).

4.4.2. Peroxidase (POX) activity

Like SOD activity, overall peroxidase (POX; EC 1.11.1.7) activity was also found to be higher in root tissues (Fig. 4b). Moreover, such activity increased in response to water stress and exogenous ABA treatment. This can be correlated with the enhanced growth rate under water stress and ABA treatment where cell wall peroxidase might be involved in cleavage of polysaccharides through $OH^{\cdot}$ formation (Singh et al., 2015). However, in hypocotyls where growth was rather inhibited under water stress and ABA treatment, increased POX activity

may have some other role like stiffening of cell wall (Passardi et al., 2005; Singh et al., 2015).

4.4.3. Ascorbate peroxidase (APX) activity

Cellular hydrogen peroxide is also scavenged by ascorbate peroxidase (APX; EC 1.11.1.1), activity of which is not only restricted to chloroplasts, but also extended to cytosol, mitochondria and peroxisomes for detoxification of H_2O_2 (Shigeoka et al., 2002). APX has been reported to be one of the important antioxidant enzymes involved in scavenging stress-induced ROS (Shigeoka et al., 2002). In *Vigna radiata* seedlings significant level of APX activity was observed not only in shoot tissues (chloroplastic) but also in root tissues (Fig. 5a). Moreover, roots under water stress showed somewhat increased APX activity that indicates its role in reducing oxidative load due to water stress-induced ROS generation. However, a rather decline in APX activity in roots under ABA treatment is not clear. On the other hand, in case of shoot, lower APX activity in water stress grown shoot tissues might be implicated for growth inhibition due to less scavenging of ROS under water stress. ABA has possibly no significant role in scavenging ROS through APX activity, as ABA treated shoot (hypocotyls) showed more or less similar activity as compared to control (Fig. 5a).

4.4.4. Catalase (CAT) activity

Catalase (CAT; EC 1.11.1.6), that dismutates H_2O_2 into water and molecular O_2, is predominantly present in peroxisomes and other cell compartments (Simova-Stoilova et al., 2009) and also involved in elimination of H_2O_2 in the mitochondria and microbodies (Shigeoka et al., 2002) thus ameliorating oxidative stress. In *V. radiata* seedlings higher catalase activity particularly in roots under PEG-induced water stress and exogenous ABA treatment (Fig. 5b) suggests the involvement of CAT activity primarily in ROS management during water stress possibly through mediation of ABA.

4.5. Conclusion

Finally, it may be concluded that during early seedling growth of *Vigna radiata* mild water stress promotes root growth while retarding shoot (hypocotyl) growth, which is probably effected by ABA accumulated in both the organs in response to water stress having differential action. The promotion of root growth is possibly mediated via NADPH oxidase generated apoplastic ROS that also signals for induction of antioxidant enzymes activities. Inhibition of hypocotyl growth by water stress is also mediated by ABA but does not involve ROS.

Contributions

SD and RKK conceived and designed the work. SD performed the research work. SD and RKK analysed the data. The manuscript was written by SD and RKK and approved by both the authors.

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